

Studies on Enzyme Action. XIII.—Enzymes of the Emulsin Type.

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D 1850 Phaseolunatin—a β -glucoside. Hydrolysis by enzymes.

D 6300 Hydrogen cyanide. Estimation of

D 8014 } Enzymes of the emulsin type. Action on Fischer's glucoside, amygdalin
Q 1240 } and phaseolunatin.

D 8014 Cellase. Behaviour towards cyanophoric glucosides of.]

In the previous communication of this series (No. XII, 1908, B, Vol. 80, pp. 321—329*) the contention was advanced that "emulsin" prepared from sweet almonds contains at least three distinct enzymes, viz., *amygdalase*, a β -*glucase* and *gluco-lactase*: the first of these, amygdalase, being the enzyme by which amygdalin is resolved into glucose and Fischer's glucoside, β -glucase that by which β -glucosides are hydrolysed, gluco-lactase that which hydrolyses milk sugar.

Meanwhile the striking discovery has been made by Rosenthaler† that emulsin has the property of inducing the formation of dextro-rotatory benzylidenecyanhydrol—such as may be obtained from amygdalin—from benzaldehyde and hydrogen cyanide; this raises the question whether there may not also be present in "emulsin" a specific enzyme capable of operating on the cyanophoric radicle of amygdalin and of resolving it into hydrogen cyanide and benzaldehyde. To avoid periphrasis this hypothetical enzyme may be spoken of as *benzcyanase*.

In continuing the study of the problem presented by almond "emulsin," we have been led to contrast it with active preparations producing somewhat similar effects derived from other sources, our hope being that we should sooner or later find one or other specific enzyme occurring alone. One of the preparations which has attracted our special attention is that derived from *Phaseolus lunatus*, the seed of which, as Dunstan and his co-workers have shown, contains a cyanophoric glucoside that is resolved under the influence of the enzyme present in the beans into glucose, acetone and hydrogen

* *Correction, paragraph 4, p. 325 : for β -methylglucoside read α -methylglucoside.*

† 'Biochemische Zeitschrift,' 1908, vol. 14, pp. 238—253; 1909, vol. 17, pp. 257—269.

cyanide and is therefore allied to Fischer's glucoside in the manner shown in the following formulæ:—



The statements made with regard to this glucoside and the associated enzyme by Dunstan, Henry and Auld in their several communications to this Society* are of a very striking character.

In their valuable summary account of the cyanophoric glucosides, read at the meeting of the British Association in York, in 1906,† Dunstan and Henry point out that the enzymes found in *P. lunatus* seeds are of special interest. To quote almost their words:—

The mixture prepared from the beans contains at least two enzymes, one of the emulsin type and the other of the maltase type; it is the latter which attacks phaseolunatin. Examination of the dextrose produced by the hydrolysis of phaseolunatin by the maltase-like enzyme present in the beans shows that it is the α -isomeride, so that phaseolunatin is an α -glucoside and may therefore be represented as the α -dextrose ether of acetonecyanohydrin.

In order that these statements may be understood, it is desirable to give a short account of the work on which they are based. It appears that the glucoside termed *phaseolunatin* by Dunstan and Henry, by whom it was described in 1903, was first isolated, in 1891, from young flax plants, by Jorissen and Hairs, by whom it was termed *linamarin*—a name for which, therefore, priority may be claimed. The statement made by Dunstan and Henry, in 1903, was that the enzyme of *Phaseolus lunatus* readily hydrolyses amygdalin, salicin and phaseolunatin. As the latter was also hydrolysed by the emulsin of sweet almonds, it was probable that the enzyme of *Phaseolus lunatus* is emulsin.

Jorissen and Hairs, who examined the enzyme contained in flax seedlings, found—to quote Dunstan—that whilst it had the property of hydrolysing both linamarin and amygdalin, the emulsin of almonds was incapable of decomposing linamarin.

Jouck, however, states, in opposition to Jorissen and Hairs, that linamarin is decomposed by the emulsin of almonds.

The enzyme extracted from flax seeds was found by Dunstan, Henry and Auld to have a range of activities similar to that of the emulsin of almonds: it readily hydrolysed amygdalin and salicin.

They were led to conclude that probably the same enzyme is contained in *Phaseolus lunatus* seed and in flax seed and that this enzyme is of the emulsin type—i.e. it appears to hydrolyse β -glucosides—and exhibits similar activities; but they recognised that it presented certain well-marked differences from emulsin.

These differences were clearly defined in a later communication, in which they withdrew the statement that phaseolunatin is decomposed by emulsin of almonds, explaining that this had been made on the basis of “a single experiment in which prussic acid was undoubtedly liberated when a commercial emulsin preparation was added to an aqueous solution of the glucoside. This experiment has, however, been repeated frequently, using several different commercial emulsin preparations as well as emulsin prepared by ourselves from sweet almonds and in no case have we been able to observe again the formation of prussic acid within a reasonable time, though in every case prussic acid

* Dunstan and Henry, ‘Roy. Soc. Proc.’ 1903, vol. 72, pp. 285—294; Dunstan, Henry and Auld, *ibid.*, B, 1906, vol. 78, pp. 145—152; Dunstan, Henry and Auld, *ibid.*, B, 1907, vol. 79, pp. 315—322.

† Dunstan and Henry, B. A. Report, 1906, p. 150.

was liberated on the further addition of the mixture of enzymes prepared from the beans of *Phaseolus lunatus* or from flax or cassava."

Finding that almond emulsin decomposed amygdalin and salicin but not phaseolunatin, whilst the "enzyme" associated with this last glucoside affected all three, they were led to think that perhaps two enzymes were present in *Phaseolus* beans, etc.; the experiments they then made led them to the conclusion that this was the case and that the one enzyme was of the emulsin type, the other of the maltase type. In fact, as before stated, they were led to think that phaseolunatin is an α -glucoside.

The evidence on which this conclusion was based was twofold: in the first place, in comparative experiments with methyl- α -glucoside and phaseolunatin, they found that both compounds were hydrolysed in contact with yeast extract and still more readily when the solution was digested with dried yeast. This led them to apply the method developed by E. F. Armstrong, involving the determination of the forms in which dextrose is liberated on hydrolysing the glucosides by noticing the rotatory power of the solution and then adding sufficient alkali to determine the rapid formation of an equilibrated mixture of the two isomeric forms of glucose.

Finding that alkali caused an increase in the laevorotatory power, they came to the conclusion that glucose was liberated in the α -form.

The conclusion finally arrived at was that the extract prepared from *Phaseolus* beans contains an α -enzyme which if not identical with yeast maltase is of the same type; and that it also contains a β -enzyme identical with or similar to emulsin. These conclusions were arrived at, it should be mentioned, in face of the known fact established by Henry and Auld that yeast contains an emulsin-like enzyme.*

The results arrived at by Dunstan and his co-workers are so remarkable, it is so difficult to reconcile their views in all respects with existing opinion, they cross our field of investigation in so many ways, that we have felt compelled to include the *Phaseolus* glucoside and its attendant enzyme in our programme of work. The results we have arrived at are such as to lead us to conclusions very different from those we have referred to.

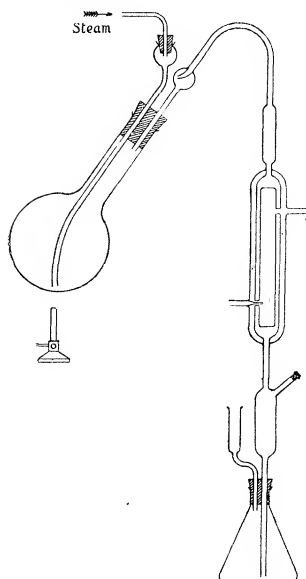
In our opinion, the glucoside extracted from *Phaseolus lunatus* seeds is not an α -glucoside and we are convinced also that the hydrolyst associated with it is not of the α -type; the evidence appears to us to be such as to justify the conclusion that both the glucoside and the correlated enzyme belong to the β -series.

Stress may be laid on the fact that in all the experiments described (except some of those with methyl α -glucoside) the concentration of the glucoside in the solutions studied was initially one-fifth molecular. We are strongly of the opinion that concordance will not be apparent between the results of different workers, even when using the same hydrolytes and the same enzymes, until agreement be arrived at to carry out experiments under "molecularly" comparable conditions. The concordance which probably exists between apparently conflicting results published by different authors is, we think, often hidden by the differences in the conditions under which observations are made.

* 'Roy. Soc. Proc.,' 1905, B, [vol. 76, p. 568.]

The Estimation of Hydrogen Cyanide.—In most cases the determination of the activity of the enzyme preparations considered in this communication has involved the estimation of hydrogen cyanide; the method we have adopted is a simplification of that referred to in No. XII of these studies (p. 329). Instead of filtering off the precipitate of silver cyanide in the manner there described, after running the measured sample (10 c.c.) into a flask (300 c.c.) containing the solution of silver nitrate (15 c.c.—N/5) and then adding sodium acetate (15 c.c.—N/5), if benzaldehyde be present we pass steam into the mixture during 2—3 minutes until the aldehyde is volatilised and then add to the cooled liquid, ammonia (5—10 c.c.) and afterwards a solution of sodium chloride (4 c.c.—N/1).

The flask is then connected to a condenser and excess of a saturated solution of tartaric acid (about 20—25 c.c.) is poured in through a thistle funnel (see figure). The liquid is



heated to the boiling point and a current of steam passed in during 15 minutes, the distillate being collected in a slight excess of an N/5 solution of caustic potash. The distillate is titrated with an N/20 solution of silver nitrate. An estimation can be effected in 30 minutes. The excessive frothing which often occurs when the liquid is distilled may be prevented by adding a few drops of olive oil.

To compare the method with the modification of Fordos and Gelis' method adopted by Dunstan, Henry and Auld, amygdalin was hydrolysed by emulsin and the amount of hydrogen cyanide liberated was then estimated by both methods.

A solution of 20·451 grammes of amygdalin in 200 c.c. of water containing 10 c.c. of the solution of emulsin was kept at 25°. At intervals of (usually) an hour 22 c.c. were withdrawn and introduced into a flask containing a drop of concentrated sulphuric acid, to stop the action of the enzyme. From this sample two portions, each 10 c.c., were taken: in one the cyanide was estimated as described above; the other was added to excess of a solution of sodium hydrogen carbonate and either titrated directly with an N/50 solution of iodine, or it was diluted and an aliquot portion titrated. The following results were obtained:—

Time.	Volume (c.c.) of		Weight (gramme) of prussic acid.		Percentage of hydrolysis.	
	Silver solution.	Iodine solution.	From silver.	From iodine.	From silver.	From iodine.
Hours.						
1	4·15	40·9	0·01127	0·01143	20·9	21·1
2	6·95	66·0	0·01888	0·01844	34·9	34·1
3½	9·6	91·5	0·02608	0·02557	48·3	47·3
4	10·7	94·5*	0·02907	0·02641	53·8	48·9*
5	11·95	105·0*	0·03247	0·02935	60·1	54·3*
6	—	111·5*	—	0·03116	—	57·7*
24	18·3	174·0	0·04972	0·04863	92·0	90·0

* In these cases the titrations were carried out on the following day; the results are given as showing that it is necessary to titrate at once with iodine to obtain correct values.

A second experiment, carried out with the same quantities of glucoside and enzyme under similar conditions, gave the following results :—

Time of action.	Volume (c.c.) of		Weight (gramme) of prussic acid.		Percentage of hydrolysis.	
	Silver solution.	Iodine solution.	From silver.	From iodine.	From silver.	From iodine.
Hours. 1	3·7	37·3	0·01005	0·01021	18·6	18·9
		(a) (b)		(a)		(a)
2	6·25	61·5 60·0	0·01698	0·01683	31·4	31·1
3	8·25	81·0 80·5	0·02241	0·02217	41·5	41·0
4	9·8	95·5 94·5	0·02663	0·02614	49·3	48·4
5	11·1	107·5 105·5	0·03016	0·02942	55·8	54·4
6	12·2	118·5 116·0	0·03315	0·03243	61·3	60·0
7	13·0	129·25 127·5	0·03532	0·03537	65·5	65·5
24	17·45	174·4	0·04741	0·04773	87·7	88·3

The titrations giving the values marked (a) in the third column were effected in the absence of starch, those marked (b) in its presence. It appears that this indicator gives erroneous results when used under conditions such as were observed by us. We noticed, in fact, that the coloration produced was very evanescent and that, unless the first appearance of a colour which did not vanish immediately on shaking were taken as indicating the end of the change, no definite result could be obtained. In all the experiments, except the first in the first table and the first and last in the second, an aliquot part of the sample was titrated with iodine. As the experiments in which the whole of the solution was titrated are also those in which the results afforded by the iodine method are higher than the results on using silver nitrate, it would appear that there is some connexion between the two facts. We do not, however, understand why this should be the case.

We are led by our experiments to conclude that in estimating small amounts of hydrogen cyanide such as were dealt with by Dunstan, Henry and Auld their method is preferable; but that in estimating the much larger amounts dealt with by us, the silver method may be used with equal advantage. The iodine method has the disadvantage that the titrations to be of any use must be effected immediately the sample is taken; this necessity restricts the use of the method to the daytime, unless starch be used as an indicator: as we have stated, this has given erroneous results in our hands.

Action of the "Enzyme" from Almonds on the Phaseolus Glucoside.—Our experiments show that the glucoside is *invariably hydrolysed* by almond-emulsin, although to a very minor extent; the conflicting results of different workers must be attributed to the use of enzymes of varying degrees of activity. In our experience, commercial emulsin is a material of variable activity; it is usually weaker than the extract prepared in the manner described in our previous communication.

Equivalent M/5 solutions of the Phaseolus glucoside and of amygdalin, containing per 100 c.c. 20 c.c. of a solution of almond-emulsin (No. XII, p. 324) were hydrolysed, the one to the extent of 2·25 per cent., the other to

the extent of 94 per cent., in 24 hours at 25°. At 37° the solution of the Phaseolus glucoside was hydrolysed to the extent of only 4·5 per cent. in 24 hours and of 16·5 per cent. at the end of seven days; apparently, the almond extract contained a very minute proportion of enzyme capable of hydrolysing this glucoside.

Action of the Phaseolus Enzyme on Amygdalin.—A fact not brought out by any previous observer is that the enzyme extracted from Phaseolus seeds* acts but slightly on amygdalin.

A solution (M/5) of amygdalin, containing 20 c.c. of a solution of the enzyme prepared from Phaseolus seeds was hydrolysed only to the extent of 2·5 per cent. in 24 hours at 25°, whilst 4 per cent. underwent change in 24 hours at 37° and 12 per cent. in seven days.† A similar solution of an equivalent amount of the Phaseolus glucoside was hydrolysed by the correlated enzyme to the extent of 50·5 per cent. in 24 hours at 25° and of 77·5 per cent. in the same time at 37°.

If, therefore, the view that the Phaseolus enzyme contains both an α - and a β -glucase be accepted, this remarkable reciprocal behaviour of the two enzymes to the two hydrolytes must be taken as an indication that almond-emulsin also contains both an α - and a β -enzyme; moreover, that the relative proportions in which the two forms are present are reversed in the two enzymes. On this supposition however, as almond-emulsin is without action on methyl- α -glucoside, the α -enzyme it contains must be different from that present in yeast maltase!

Action of the Phaseolus Enzyme on Methyl- α -glucoside and on Maltose.—From the statement made by Dunstan, Henry and Auld that yeast maltase decomposes methyl- α -glucoside and maltose more rapidly than does the enzyme of *Phaseolus lunatus*, it is to be inferred that these two compounds are hydrolysed by the Phaseolus enzyme. We have not been able to detect any trace of action, even when the substances were allowed to remain in contact during several weeks.

In a preliminary experiment in which an M/5 solution of methyl- α -glucoside containing 20 c.c. of phaseolunatase solution per 100 c.c., was heated at 25°

* Our preparation was made from ground Java beans, some of which were black, others various shades of brown and a few white. The precipitate produced by alcohol in the aqueous extract, which had been exposed over potash to remove hydrogen cyanide, was shaken up with about six times its weight of water.

† Experiments have been recorded by Auld ('Chem. Soc. Trans.,' 1908, vol. 93, p. 1266) which appear to show that the Phaseolus enzyme acts somewhat readily on amygdalin—to the extent of as much as 34·5 per cent. within an hour.

[The discordance between this result and our own is considerably reduced if allowance be made for the different proportions of the materials used; nevertheless, the value is in advance of any we have obtained.—April 9.]

during 24 hours, the initial optical rotation was $9^{\circ}75$ and the final value $9^{\circ}78$. An experiment was then made in the following manner:—25 c.c. of a 2M/5 solution of methyl- α -glucoside having been measured into each of three 50-c.c. Jena flasks and 20 c.c. of water into one, 10 c.c. into the second but none into the third, 5 c.c. of the phaseolunata solution was added to the first, 15 c.c. to the second and 25 c.c. to the third. Three other solutions were prepared in the same way but from a solution of the glucoside only half as strong as that first used. From each of these six mixtures a sample of 10 c.c. was withdrawn immediately after adding the enzyme and transferred to a 50 c.c. measuring-flask containing a drop of a solution of sodium hydroxide to stop the action of the enzyme. All the operations were carried out with the liquids at 25° . The main bulk of the solutions was kept at 25° during 14 days and then at 37° during a further 14 days. At intervals of seven days, of 14 days and of 28 days, a sample measuring 10 c.c. was removed from each solution and the action stopped by soda. Each sample was diluted to 50 c.c. and its reducing power was then determined by the method described by Brown, Morris and Millar ('Chem. Soc. Trans.,' 1897, vol. 71, p. 278). The following table gives the weights of copper reduced by 20 c.c. of each of the diluted samples:—

Concentration of the glucoside.	Volume of enzyme solution per 100 c.c. of acting liquid.	Weight (gramme) of copper.			
		Initial sample.	Sample after 7 days at 25° .	Sample after 14 days at 25° .	Sample after 14 days at 25° and 14 at 37° .
M/5	10	0·0037	0·0026	0·0022	0·0012
M/5	30	0·0006	0·0016	0·0006	0·0001
M/5	50	0·0007	0·0004	0·0001	0·0004
M/10	10	0·0026	0·0016	0·0009	0·0001
M/10	30	0·0010	0·0006	0·0008	0·0004
M/10	50	0·0002	0·0026	0·0001	0·0001

As a correction involving the deduction of 0·0035 gramme of copper has to be made in each case, on account of the reduction which takes place when the Fehling's solution alone is carried through the series of operations involved in making the determinations, it is obvious that not the slightest amount of methyl- α -glucoside was hydrolysed.

It might be urged that the exposure of the enzyme at a temperature of 37° during so long a period as 14 days would be likely to diminish its activity considerably, if not to destroy it; consequently that the prolongation of the experiments over so long a time proves little or nothing. To decide this point, 5 c.c. was withdrawn from each of the six flasks, each sample being

put into a separate 30-c.c. flask; to each flask was then added 5 c.c. of an M/2 solution of phaseolunatin and 2.5 c.c. of water, thus giving six M/5 solutions of phaseolunatin containing respectively 4 c.c., 12 c.c., 20 c.c., 4 c.c., 12 c.c., and 20 c.c. of the original solution of phaseolunatin per 100 c.c. These solutions were maintained at 25° during 24 hours and the amount of hydrolysis effected was then determined by estimating the amount of hydrogen cyanide liberated. The percentages of phaseolunatin decomposed were as follows:—

Volume (c.c.) of original enzyme solution present per 100 c.c.	Percentage of glucoside hydrolysed.
4	9.0
12	28.25
20	46.5
4	6.0
12	29.0
20	40.7

As an M/5 solution of phaseolunatin containing 20 c.c. of the same phaseolunatin solution per 100 c.c. was hydrolysed to the extent of 45.25 per cent. (*i.e.* 2.26 per cent. per 1 c.c. per 100 c.c.) in 24 hours at 25°, it is evident that the enzyme was practically unaffected by contact with M/5 or M/10 methyl- α -glucoside solution during 14 days either at 25° or at 37°.

Action of Phaseolunatin on Maltose.—In a preliminary experiment the reducing power of an M/5 solution of maltose, containing 10 c.c. of the solution of the phaseolunatin per 100 c.c., was determined initially and after 24 hours at 25°

2 c.c. of the liquid reduced initially 0.1568 gramme of copper.

2 c.c. „ „ finally 0.1509 „ „

Since any hydrolysis of the maltose would increase the reducing power of the solution, it must be concluded that none had taken place.

An attempt was made to carry out a series of experiments similar to that with methyl- α -glucoside. It was impossible, however, to filter off the reduced cuprous oxide in any reasonable time, owing to the clogging of the filter by the precipitated proteid; so the experiments had to be abandoned.

It appears to us that the mistake Dunstan, Henry and Auld made was to assume that they were dealing with yeast maltase and to overlook the possible presence in their yeast of an enzyme capable of hydrolysing β -glucosides.

Action of Yeast Extract on the Phaseolus Glucoside.—The proof that the Phaseolus enzyme is without action on methyl- α -glucoside and on maltose is sufficient to show that it is neither α -glucase nor maltase; it is nevertheless

conceivable that yeast has an action on the glucoside in virtue of the presence of *amygdalase*, the enzyme characterised by Caldwell and Courtauld, although this is in no way probable in view of the fact that the *Phaseolus* enzyme has but little action on amygdalin. The existence of this enzyme was not yet recognised at the time when Dunstan, Henry and Auld were led to conclude that the *Phaseolus* glucoside is an α -derivative.*

Although the experiments we have made with heated and unheated extracts from various yeasts have given very irregular results, it appears to us that they afford conclusive evidence that phaseolunataase is not amygdalase. As often as not, little or no action was noticeable, whilst in some cases 5 or 6 per cent. at most of the *Phaseolus* glucoside was hydrolysed within 24 hours at 25°: any action observed in such cases we are inclined, therefore, to attribute to the presence of a minute proportion of "emulsin."

In preparing Fischer's glucoside from amygdalin by means of yeast, some hydrogen cyanide is always liberated, the yield of the glucoside being by no means satisfactory.

On one occasion an exceptionally strong extract prepared by digesting 20 grammes of a particular fresh yeast with 100 c.c. of water was found to

* Auld has made the assumption that amygdalase is an α -enzyme and has described experiments which he assumes afford proof that the glucose liberated when amygdalin is resolved into Fischer's glucoside and glucose is the α -form and that liberated from Fischer's glucoside the β -form. In our opinion, his argument is rendered invalid by the fact that both amygdalin and Fischer's glucoside are "racemised" somewhat rapidly by alkali; E. F. Armstrong's method, therefore, cannot well be applied to these materials.

The following observations are quoted as showing the extent to which change takes place. A solution of mandelonitrile-glucoside contained in a 2-decimetre jacketed polarimeter tube kept at 25° having been mixed with a drop of 3N ammonia, observations of the optical rotation were taken at minute intervals.

Before the addition of alkali the rotation was.....	— 1°77
1 min. after the addition of alkali the rotation was	— 1°85
2 mins. " " " " "	— 1°93
3 " " " " "	— 2°03
4 " " " " "	— 2°12
5 " " " " "	— 2°21
6 " " " " "	— 2°27
7 " " " " "	— 2°35
8 " " " " "	— 2°42
9 " " " " "	— 2°47
10 " " " " "	— 2°53
30 " " " " "	— 3°23

Three comparative experiments were also made by taking equivalent solutions of amygdalin, mandelonitrile-glucoside and ordinary (α) glucose; after observing the optical rotatory power, each was mixed with the same proportion of alkali (one drop of 3N ammonia) and the change of rotation determined when all three solutions were kept

effect the decomposition of no less than 13·5 per cent. of the glucoside; although very active towards methyl- α -glucoside, this extract had no action on amygdalin, a quite unusual circumstance. On repeating the experiment several months later with the same yeast but with an extract prepared by digesting only 15 grammes with 300 c.c. of water, no action was observed either on phaseolunatin or on amygdalin.

The probability that yeast contains a " β -glucase" is considerable in view of the fact that cellulose is now known to be a compound of the β -glucoside class; cytase (cellase), if not " β -glucase," is probably a closely allied enzyme.

It is clear, however, that not only do different yeasts yield different enzymes or different proportions of the several enzymes, but also that age, as well as race and the conditions under which the yeast is dried and extracted, are all factors to be taken into account as influencing the character of the extract.

Nature of the Dextrose Residue in Phaseolunatin.—Dunstan, Henry and Auld give the following description of their experiments made with the object of characterising the glucose produced by the hydrolysis of phaseolunatin :—

"To a solution of 3 grammes of the glucoside in 20 c.c. of water, 0·5 gramme of the enzyme preparation was added and the mixture kept at 40° in a stoppered bottle. For each observation a few cubic centimetres of the liquid were withdrawn, mixed with a small

under observation at the ordinary temperature (15°). The following are the results obtained :—

	Glucose.	Mandelonitrile glucoside.	Amygdalin.
	°	°	°
Initial rotation	+ 3·89	− 1·91	− 3·83
Rotation 1 minute after adding alkali.....	+ 3·57	− 1·93	− 3·84
" 2 minutes " " 	+ 3·32	—	− 3·88
" 3 " " " 	+ 3·06	− 2·03	− 3·91
" 4 " " " 	+ 2·89	− 2·10	− 3·94
" 5 " " " 	+ 2·73	− 2·15	− 3·95
" 6 " " " 	+ 2·63	− 2·18	− 3·98
" 7 " " " 	+ 2·53	− 2·21	− 4·02
" 8 " " " 	+ 2·48	− 2·22	− 4·05
" 9 " " " 	+ 2·37	− 2·25	− 4·06
" 10 " " " 	+ 2·33	− 2·31	− 4·08

From these figures, it is obvious that the change undergone by glucose is more rapid than that undergone by mandelonitrile-glucoside and by amygdalin; nevertheless, the extent to which racemisation of the latter two substances takes place in the time (according to Lowry about 12 minutes in the presence of N/100 alkali such as we used) required to effect the complete muta-rotation of glucose is too great to permit the application of E. F. Armstrong's method in the presence of the two cyanophoric glucosides.

definite quantity of alumina cream and immediately filtered through a closely-felted pad of asbestos. The optical rotation of the clear filtrate was then determined *before* and *after* addition of a drop of ammonia solution, the latter being used, as indicated by Lowry, to establish equilibrium. The same polarimeter tube was used throughout the experiments."

Time of action.	Initial rotation.	Rotation after addition of ammonia.	Change in rotation.
Hours.	° /	° /	° /
0	-1 15	-1 15	0 0
1	-0 46	-1 11	-0 25
2	-0 35	-0 58	-0 23
3	-0 34	-0 57	-0 23
20	-0 56	-0 56	0 0

With reference to these figures, it may be noted that a solution of 3 grammes of phaseolunatin in 20 c.c. of water will have an optical rotatory power of $-4^{\circ}11$ (approximately); in order to reduce this to the observed $-1^{\circ}25$, the solution must have been treated with almost three times its volume of alumina cream, supposing the observations were made in a 1-decimetre tube; such an amount can scarcely be described as "small."

Secondly, if we are to assume that the same proportion of alumina cream was always used—and otherwise we do not understand the meaning of the word "definite"—the changes in rotation given in the fourth column above are far larger than can possibly have been produced by the muta-rotation of glucose. The decomposition of the phaseolunatin contained in a 50-per-cent. solution (the optical rotation of which in a 2-decimetre tube is identical with the specific rotatory power of the glucoside) into hydrogen cyanide, acetone and α -glucose would involve a change in the rotatory power of 104° , $27^{\circ}4$ being consequent on the destruction of the glucoside and $76^{\circ}6$ on the liberation of α -glucose. Assuming that the glucose set free does not change spontaneously, any decrease in the lævo-rotatory power of a solution of phaseolunatin which is undergoing hydrolysis will be one-fourth due ($27\cdot4/104$ exactly) to the disappearance of glucoside and three-fourths to the liberation of α -glucose. The initial rotation is given as $-1^{\circ}15'$ and that after one hour as $-0^{\circ}46'$, thus showing a fall in lævo-rotation of $29'$. Of the latter, as indicated above, only three-fourths or approximately $21'$ can be due to the production of α -glucose. The muta-rotation of glucose causes a change in the specific rotatory power from 105° to $52^{\circ}7$, that is to say roughly a decrease of 50 per cent.; hence the fall of $21'$, due to the liberation of α -glucose, will be reduced to $10'\cdot5$ on adding alkali, that is to say to a change of $10'\cdot5$ (roughly), whereas that observed was $25'$. The same argument can be applied to the other values given.

On reference to E. F. Armstrong's account of his work* it will be found that his original observations are, in most cases, affected by similar "errors." It would seem that the changes taking place in the solutions examined are not only those which are involved in the alteration of the glucose originally produced into the equilibrated mixture of isomerides—in fact, the substances present in the solution of the enzyme are also affected by the alkali.

Our experiments have afforded abundant evidence that such is the case, as will be pointed out later on. Moreover, we find that the Phaseolus enzyme is itself sensitive to alkali; thus a solution of which the initial rotatory power was $+0^{\circ}01$ was found to be considerably altered, the rotatory power changing from $-0^{\circ}02$, the value after adding alkali, to $-0^{\circ}95$ at the end of four hours.

Under these circumstances we have deemed it desirable to repeat some of E. F. Armstrong's experiments, in order to verify his statements—especially as the instrumental appliances at our disposal are far better than those over which he had command. The following series of observations made with methyl- β -glucoside and emulsin are a complete justification of his method:—

Time of action.	Initial rotation.	Rotation after addition of ammonia.	Change in rotation.
hours.	°	°	°
0	-2·83	-2·80	+0·03
1	-2·30	-2·14	+0·16
1·5	-2·03	-1·85	+0·18
2	-1·74	-1·59	+0·15
2·5	-1·53	-1·38	+0·15
4	-0·71	-0·61	+0·10
4·5	-0·54	-0·44	+0·10
6	-0·12	-0·04	+0·08
24	+2·63	+2·62	-0·01

Attempts to carry out a similar experiment with maltose were unsuccessful owing to our failure to obtain enzyme extracts which remained clear on the addition of alkali. It may be mentioned that in E. F. Armstrong's experiments the liquid was filtered to remove the turbidity occasioned by alkali; we have sought to avoid any such operation and have added the alkali directly to the liquid in the tube.

An M/5 phaseolunatin solution containing 25 c.c. of phaseolunatase solution per 100 c.c. was kept in a Jena flask at 37° . At hourly intervals, 20-c.c. samples were withdrawn, mixed with 5 c.c. of alumina cream, rapidly filtered through asbestos and examined polarimetrically at 25° in a 2-decimetre tube. Then two drops of soda solution were added and the rotation again determined.

* 'Chem. Soc. Trans,' 1903, vol. 83, p. 1305.

Time of action.	Initial rotation.	Rotation after addition of soda.	Change in rotation.
hours.	°	°	°
0	-3·40	-3·36	+0·04
1	-3·12	-3·11	+0·01
2	-2·83	-2·80	+0·03
3	-2·57	-2·55	+0·02
3·5	-2·43	-2·43	0·0
5	-2·10	-2·11	-0·01
6	-1·80	-1·81	-0·01
7	-1·65	-1·65	0·0
8	-1·51	-1·50	+0·01
25	-0·27	-0·21	+0·06

The differences observed in this case were too small to be of any positive value. Further experiments were carried out as follows:—

A solution of 7·4143 grammes of phaseolunatin in water occupying 75 c.c. at 25° was mixed with 75 c.c. of a solution of phaseolunatase, also at 25°. The latter solution was prepared by shaking 100 c.c. of the cloudy extract with 20 c.c. of alumina cream and filtering; this treatment gave a clear solution of the enzyme, highly active towards phaseolunatin. A 2-decimetre jacketed polarimeter tube was filled with the mixture of glucoside and enzyme and the rotation observed at 25°; then a drop of a solution of soda was added and the rotation again determined. Meanwhile the main bulk of the liquid (which was now an M/5-solution of the glucoside) was heated to 37° and kept at this temperature, samples being withdrawn at intervals and examined in the manner described in the case of the initial experiment. The following results were obtained:—

	Time of action.	Initial rotation.	Rotation after addition of soda.	Mean change in rotation.
	Hours.	°	°	°
1st experiment...	0	-4·24	-4·21	+0·03
	1	-3·99	-3·95	+0·04
	2	-3·57	-3·51	+0·06
	3	-3·26	-3·23	+0·03
	4·5	-2·81	-2·81	0·0
	5	-2·56	-2·55	+0·01
	6	-2·31	-2·31	0·0
	7	-2·04	-2·11	-0·07
	24	-0·27	-0·36	-0·09
2nd experiment...	0	-3·76	-3·74	+0·02
	1	-3·66	-3·65	+0·01
	1·5	-3·53	-3·50	+0·03
	2·5	-3·27	-3·26	+0·01
	4	-2·90	-2·90	0·0
	5·5	-2·61	-2·66	-0·05
	6·5	-2·45	-2·49	-0·04
	24	-0·35	-0·44	-0·09

We are of opinion that the change indicated in these various cases is in the direction corresponding with the conversion from β - into α -glucose, not from α into β . But this change is complicated by some change in the other substances present in the solution which somewhat masks that undergone by the glucose.

The complete set of readings obtained in the course of another experiment may be given with advantage, as illustrating the procedure and also because of the evidence they afford that changes are proceeding such as have been referred to. The equilibration of the glucose by means of the alkali added (1 drop of a 10 N solution) is practically an instantaneous process. It should be mentioned that the set of readings given on each of the lines in the table would occupy about five to six minutes, readings being taken at intervals of about half a minute.

Time.	Polarimeter readings.	Mean rotation.	Change in rotation.
1 hour	{ Initial $-4^{\circ}20$, 21, 23, 23, 20, 22, 23, 20, 23, 22 + alkali $-4^{\circ}18$, 20, 17, 21, 14, 13, 18, 18, 17, 14	{ $-4^{\circ}22$ $-4^{\circ}17$	+ $0^{\circ}05$
1.5 hours ...	{ Initial $-3^{\circ}96$, 97, 96, 95, 95, 94, 95, 96, 94, 95 + alkali $-3^{\circ}91$, 90, 94, 94, 93, 93, 89, 89, 94, 89	{ $-3^{\circ}95$ $-3^{\circ}91$	+ $0^{\circ}04$
2 " ...	{ Initial $-3^{\circ}69$, 69, 69, 67, 70, 66, 68, 69, 68, 68 + alkali $-3^{\circ}64$, 64, 68, 69, 67, 67, 67, 67, 67, 67	{ $-3^{\circ}68$ $-3^{\circ}67$	+ $0^{\circ}01$
3.5 " ...	{ Initial $-2^{\circ}77$, 78, 79, 78, 79, 82, 83, 80, 81, 81 + alkali $-2^{\circ}77$, 80, 82, 87, 84, 85, 87, 89, 85, 89	{ $-2^{\circ}80$ $-2^{\circ}84$	- $0^{\circ}04$
4 " ...	{ Initial $-2^{\circ}59$, 55, 60, 55, 57, 58, 58, 57, 57, 58 + alkali $-2^{\circ}60$, 60, 60, 58, 61, 63, 67, 66, 67, 70	{ $-2^{\circ}57$ $-2^{\circ}63$	- $0^{\circ}06$
5 " ...	{ Initial $-2^{\circ}08$, 07, 08, 11, 10, 11, 10, 08, 10, 11 + alkali $-2^{\circ}07$, 13, 08, 24, 24, 18, 20, 24, 21, 23	{ $-2^{\circ}09$ $-2^{\circ}18$	- $0^{\circ}09$
6 " ...	{ Initial $-1^{\circ}44$, 43, 44, 43, 46, 46, 45, 45, 47, 46 + alkali $-1^{\circ}48$, 52, 52, 54, 53, 54, 57, 58, 55, 55	{ $-1^{\circ}45$ $-1^{\circ}54$	- $0^{\circ}09$
24 " ...	{ Initial $+0^{\circ}56$, 54, 56, 55, 54, 55, 56, 56, 55, 55 + alkali $+0^{\circ}48$, 40, 42, 42, 41, 40, 42, 40, 41, 40	{ $+0^{\circ}55$ $+0^{\circ}41$	- $0^{\circ}14$

The figures given in the last column are obtained from the mean rotations before and after the addition of alkali. If, however, the first reading after the addition of alkali be compared with the last prior to the addition—thus reducing the possible change of the enzyme to a minimum—the changes in rotation become $+0^{\circ}04$, $+0^{\circ}04$, $+0^{\circ}04$, $+0^{\circ}04$, $-0^{\circ}02$, $+0^{\circ}04$, $-0^{\circ}02$, and $-0^{\circ}07$ respectively, values which lend still more support to the conclusion that the glucose is liberated in the β -form. Moreover, the observation in all three experiments of a comparatively large change produced by adding alkali to the 24-hour sample, when presumably all the glucose is present as an equilibrated mixture, is evidence that the enzyme suffers a change (in optical rotatory power) in a direction contrary to that undergone by the liberated glucose.

Comparative Action of Emulsin and of the Phaseolus Enzyme on Fischer's Glucoside, Amygdalin and the Phaseolus Glucoside.—Assuming that the resolution

of amygdalin is primarily conditioned by amygdalase and that any β -glucose which it contains cannot operate until the diglucoside is resolved into Fischer's glucoside and glucose, the inactivity of the Phaseolus enzyme towards amygdalin may be ascribed to the absence from it of all but traces of amygdalase. From this point of view, Fischer's glucoside, salicin and methyl- β -glucoside, rather than amygdalin, are to be compared with the Phaseolus glucoside and are the appropriate test materials to use in determining whether the correlated enzyme belongs to the β class, whether indeed it may not be simple β -glucose but present in considerably smaller proportion than in emulsin.

Our observations show that methyl- β -glucoside is far less readily attacked by the Phaseolus enzyme than it is by almond-emulsin. It is well known that this glucoside is somewhat rapidly attacked by emulsin; that phaseolunatase acts on it only slowly is shown by the following figures derived from an experiment made with an M/5 solution containing 50 c.c. of the enzyme extract per 100 c.c. :—

Time of action..... {	1 day.	2 days.	7 days.	9 days.	15 days.	17 days.	33 days.
Percentage of hydrolysis	2·3	7·2	26·1	30·5	40·6	43·7	51·5

In the case of salicin, on the other hand, the disproportion in the rates of change appears to be far less marked.

To ascertain the behaviour of the three cyanophoric glucosides, experiments were made as follows: six solutions were prepared :—

(1) By mixing 15 c.c. of a 4M/10 amygdalin solution with 15 c.c. of a dilute solution (1:10) of emulsin.

(2) By mixing 15 c.c. of a 4M/10 amygdalin solution with 15 c.c. of the solution of phaseolunatase.

(3) By mixing 15 c.c. of a 4M/10 Fischer's glucoside solution with 15 c.c. of the dilute solution of emulsin.

(4) By mixing 15 c.c. of a 4M/10 Fischer's glucoside solution with 15 c.c. of the phaseolunatase solution.

(5) By mixing 15 c.c. of a 4M/10 solution of phaseolunatin with 15 c.c. of a strong solution of emulsin.

(6) By mixing 15 c.c. of a 4M/10 solution of phaseolunatin with 15 c.c. of phaseolunatase solution.

These were kept at 37°. The same solution of enzyme was used in the case of all three glucosides, except that the emulsin used with phaseolunatin was ten times as strong as that used in the other cases. After 24 hours the

amount of hydrolysis effected was determined in each case by estimating the amount of hydrogen cyanide liberated.

Hydrolyst.	Solution (30 c.c.) containing					
	3·0676 grammes amygdalin.		1·7709 grammes Fischer's glucoside.		1·4828 grammes phaseolunatin.	
	Weight hydrolysed.	Percentage hydrolysed.	Weight hydrolysed.	Percentage hydrolysed.	Weight hydrolysed.	Percentage hydrolysed.
Emulsin, 5 hours	2·162	70·5	1·655	93·5	—	—
Emulsin, 24 hours	2·5	81·5	1·595	*89·5	0·052	3·5
Phaseolunatase, 24 hours	0·107	3·5	1·533	86·0	1·334	90·0

* The lower percentage in this instance is to be accounted for by loss of hydrogen cyanide on removal of the 5-hour sample. Whilst no appreciable loss occurs at 25° through the removal of samples at intervals, this is not the case at the higher temperature.

The striking fact brought out by these experiments is the manner in which the Phaseolus enzyme acts equally well on Fischer's glucoside and phaseolunatin, although it has but little action on amygdalin. On the assumption that emulsin is relatively rich in β -glucase—far more so than the Phaseolus enzyme—and that both glucosides are of the β -type because of this similar behaviour, it is difficult to understand the inactivity of emulsin towards phaseolunatin. It is to be noted that our observations do not stand alone: Dunstan and his co-workers came to the conclusion that emulsin has no action on phaseolunatin.

It would seem that there are peculiarities either in the enzymes or in the glucosides which await discovery. That the Phaseolus enzyme should not act on amygdalin is not surprising, as it may well be destitute of amygdalase; it is, however, difficult to explain the inactivity of almond-emulsin towards phaseolunatin, in view of the activity of phaseolunatase towards Fischer's glucoside as well as towards phaseolunatin; such a result is scarcely in favour of the view that the activity of emulsin as a hydrolyst of β -glucosides is due to a simple β -glucase, *i.e.* an enzyme compatible with and influenced by glucose alone.

As bearing on this issue, the following experiments were made with pressed yeast juice kindly placed at our disposal by Dr. Harden: four solutions were prepared:—

(1) (3) By mixing 10 c.c. of a 4M/10 solution of Fischer's glucoside with 10 c.c. of the yeast juice.

(2) (4) By mixing 10 c.c. of a 4M/10 solution of phaseolunatin with 10 c.c. of the pressed yeast juice.

Solutions (1) and (2) were kept at 37°, (3) and (4) at 25°. The yeast juice was used within 30 minutes of receipt, *i.e.* within an hour of leaving Dr. Harden's hands. After 23 hours the extent to which hydrolysis had taken place in each case was measured by estimating the hydrogen cyanide liberated.

Temperature.	Solution (20 c.c.) containing			
	1·1806 grammes Fischer's glucoside.		0·9886 gramme phaseolunatin.	
	Weight hydrolysed.	Percentage hydrolysed.	Weight hydrolysed.	Percentage hydrolysed.
°				
25	0·0915	7·75	0·0049	0·5
37	0·1682	14·25	0·0098	1·0

Seven days later, two experiments were made (at 37°) to ascertain whether the yeast juice had retained its activity and also to contrast its behaviour towards Fischer's glucoside with that towards amygdalin; the results are remarkable as showing comparatively slight activity towards the latter, only 5 per cent. of amygdalin but 13·5 per cent. of the Fischer's glucoside being changed. The yeast enzyme appears to resemble almond-emulsin in its behaviour towards the Phaseolus glucoside.

Rosenthaler's observations, as already pointed out, serve to raise the question whether there may not be in almond-emulsin an enzyme or enzymes capable of affecting the cyanophoric radicle in amygdalin. (He suggests that both a synthetic and an analytic enzyme are present.)

The fact that mandelonitrile may be separated as such from amygdalin by the action of sulphuric acid of a certain degree of concentration* would appear to lend support to this contention; this and other similar nitriles have been shown by Ultée† to be far more stable compounds than was formerly supposed, so that the production of hydrogen cyanide from amygdalin is not to be set down to the mere spontaneous decomposition of the nitrile when liberated in aqueous solution.

* Walker and Krieble, 'Chem. Soc. Trans.,' vol. 95, 1909, p. 1374.

† 'Rec. Trav. Chim.,' 1909, vol. 28, pp. 1—23; 248—260.

From this point of view, it is possible that a distinct enzyme—an *acetycyanase*—is operative in separating the nitrilic radicle from phaseolunatin and that this, rather than a simple β -glucase, is the main constituent of the active product from Phaseolus beans; but if such be the case, the enzyme must be compatible with the cyanophoric radicle in Fischer's glucoside, whilst the corresponding enzyme in emulsin must be incompatible with the nitrilic member of phaseolunatin.

Although we have been able to verify Rosenthaler's statements as to the synthetic activity of almond-emulsin, we have been unsuccessful in preliminary experiments with phaseolunataase in obtaining evidence that this enzyme can induce the interaction of benzaldehyde and hydrogen cyanide. This result apparently affords additional proof that the almond and the Phaseolus enzymes are possessed of distinctive properties. The problem afforded by "emulsin" is one which can only be solved by exhaustive study of enzymes from various sources. In conjunction with Dr. E. F. Armstrong, we have for some time been engaged in developing a method of standardising the enzymes relatively to a series of reference compounds, so that a strict comparison may be made of the activities of materials from various sources.

[To contrast the behaviour of vegetable *cytase*—which may be regarded provisionally as identical with *cellase*, the enzyme by which the β -glucoside *cellobiose* is resolved into two molecules of glucose*—with that of almond emulsin and of the Phaseolus enzyme, we have ascertained the effect produced on several cyanophoric glucosides by an extract prepared by digesting ground oats with three times their weight of water during about 15 hours. A mixture of the extract with an equal volume of a 4M/10 solution of the glucoside was digested at 37° during 48 hours. The amount of change was 8 per cent. in the case of Fischer's glucoside, 5 per cent. in that of amygdalin and 4 per cent. in the case of the Phaseolus glucoside. The greater sensitiveness of this last in comparison with amygdalin, when the results are compared with those produced by emulsin, is perhaps noteworthy and suggestive.

We may also refer to results obtained with an extract of the seeds of the kidney vetch, *Anthyllis vulneraria*, which contains an enzyme of the emulsin type. To test its activity the ground seed was macerated with about four times its weight of water. An M/5 solution of the glucoside containing half its bulk of this extract was digested at 37° during 24 hours. The amount of

* Cf. E. Fischer and G. Zemplén, 'Annalen,' 1909, vol. 365, pp. 1—6; Bertrand and Holderer, 'Compt. Rend.,' 1910, vol. 150, p. 230; Pringsheim and Zemplén, 'Zeit. Physiol. Chem.,' 1909, vol. 62, p. 367.

change was 13·5 per cent. in the case of Fischer's glucoside but only 3 per cent. in that of amygdalin and 2 per cent. in that of the Phaseolus glucoside.

It can scarcely be doubted that several specific enzymes exist which may all be spoken of as β -glucases. The common factor in these presumably is a *primary member* sympathetic with the glucose member of the glucoside; the *secondary member*—in the case of the cyanophoric glucosides, the group CXY (CN)—being different in the several enzymes. We are led to infer the existence of such a primary member sympathetic with glucose in the case of almond-emulsin and in that of the Phaseolus enzyme, as these are both controlled to a marked extent by glucose, less action taking place if glucose be added in advance.

On such an assumption, it is not difficult to understand the inactivity, for example, of the Phaseolus enzyme towards amygdalin as compared with Fischer's glucoside. It may be supposed that the molecule of glucose attached to the Fischer's glucoside in amygdalin is so placed as to render association between the hydrolyst and the primary member of the hydrolyte impossible or nearly so, not only in the case of the Phaseolus enzyme but also in that of almond-emulsin, action taking place in the case of the latter only after the amygdalase, which accompanies the β -glucase, has prepared the way by removing the terminal glucose group.—March 10, 1910.]

[The 'Comptes Rendus' of March 21 (vol. 150, pp. 793—796) contains the welcome announcement by J. Giaja that by means of an extract prepared from the snail *Helix pomatia*, L., he has succeeded in separating from amygdalin a non-reducing saccharose of the trehalose type. April 9.]

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